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19. ABSTRACT (Continue on reverse if necessary and identify by block number) The goal of this multi-investigator research program is to characterize the extra-cellular slime of selected marine gliding bacteria. We are particularly interested in the role of this material in bacterial adhesion and motility. Slime produced by marine Cytophagales isolated from biofilms on various substrata will be characterized with fine structural, physiological, biochemical, immunological and rheological approaches. A model system for these studies is the relatively well characterized strain Cytophaga sp. U67.					
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PROGRESS REPORT ON CONTRACT N00014-88-K-0158

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CONTRACT TITLE: The Role of Extracellular Slime in Adhesion and Motility of Gliding Bacteria

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RESEARCH OBJECTIVE: To characterize the slime of marine gliding bacteria and elucidate the role of this extracellular material and the cell envelope in adhesion and motility.

This is an inter-disciplinary research project. W.H. Schwarz (co-P.I., Dept. of Chemical Engineering) is doing a theoretical and rheological analysis of the slime of these bacteria (see part 2 of this report). I.W. Sutherland (Dept. of Microbiology, University of Edinburgh) will perform biochemical analyses of the extracellular slime.

PROGRESS (Year 1):

The Bacteria: The gliding bacteria are a highly heterogeneous assemblage of Gram-negatives that move on surfaces in the absence of any obvious locomotory organelles and without detectable change in cell form.

We have isolated and partially characterized a variety of Cytophagales from biofilms on glass, plexiglass and tributyltin-painted substrata in marine and estuarine waters. Other species have been acquired from culture collections.

Most of our research has centered on *Flexibacter maritimus* lyl-1 isolated from flounder by D. Baxa. This strain produces significant amounts of slime as measured by increased culture medium viscosity and cell-associated polysaccharide. It demonstrates limited gliding motility on agar and glass. The bacteria adhere rather tenaciously to glass.

Non-adherent mutants of a freshwater gliding bacterium, *Cytophaga* sp. strain U67, had been isolated previously using a hexadecane/aqueous medium partitioning protocol to enrich for cells with relatively hydrophilic surface properties. This enrichment method has not been effective in selecting for non-adherent mutants of *F. maritimus*, nor has a protocol that serially enriches for bacteria that do not adhere to glass wool fibers submerged in the culture medium. We have successfully isolated several mutants with altered colony morphology and motility by UV mutagenesis and visual screening of the colonies of surviving bacteria. Some of these strains appear to be distinct in the nature and/or amount of slime that they produce. For example, two mutants produce rough, apparently non-slimy colonies on agar. In liquid

culture, they are hyper-slime producers. These are currently being characterized.

Slime: The slime produced by gliding bacteria may have several roles including mediation of adhesion to substrata and ancillary function(s) in the mechanism of motility. The few extant studies on the slime of terrestrial and aquatic gliding bacteria indicate that the polymers that comprise this material are shed into the growth medium or deposited on the substrata on which the bacteria are gliding. Since shake cultures of some strains of *F. maritimus* produce macroscopic bacterial aggregates, our assays of slime production have until recently concentrated on shallow liquid, static cultures in which the bacteria are able to adhere to and glide on glass surfaces. Most of the extracellular polysaccharide produced under these growth conditions is firmly bound to the cell, apparently in the form of a capsule. We are currently exploring the use of anti-slime antibody to stabilize this extra-cellular material for fine structure examination.

Numerous methods have been tried to remove the bound polysaccharide by non-destructive means to permit future rheological studies on the material as well as biochemical analysis. These include manipulation of ionic conditions, use of chaotropic ions, protease treatment, shearing, ultrasound, ultracentrifugation, starvation, spheroplasting and osmotic shock. None of these methods has removed a significant proportion of the polysaccharide. We have successfully solubilized the polysaccharide with harsher treatments (eg. 100°C, NaOH, and phenol extraction). These slime extracts have been run on SDS polyacrylamide gels and stained for polypeptides. Each preparation method yields a different spectrum of polypeptides associated with (or contaminating) the released polysaccharide.

Preliminary, very recent results indicate that polysaccharide is released into the medium in shake cultures that are characterized by high surface area: volume ratios.

Adhesion Studies: We have developed several methods for quantitating the adhesive properties of these gliding bacteria and mutants in slime production. The first of these is a variation on the protocol of counting the number of bacteria adhering to coverslips submerged for a fixed time in the bacterial suspension. A second, very simple assay that provides an indication of the tenacity of adhesion, involves loading of a bacterial counting chamber (Petroff-Hausser) with cell suspension. After incubation to permit bacteria to adhere to the glass surfaces, the medium and suspended cells are flushed out by displacement with sterile medium (applied at one end of the chamber); a wick is placed at the other end. Only bacteria whose adhesion resists the flushing remain in the chamber.

The third approach for quantitating bacterial adhesion involves loading test bacteria into a rectangular cross-section micro-capillary whose optical properties permit clear visualization of adherent cells by phase contrast microscopy. We are currently assessing the tenacity of adhesion of bacteria attached to the inner surfaces by subjecting them to:

- a. centrifugal force. The capillaries are sealed at one end, mounted in an adapter in a swinging bucket rotor, and centrifuged.
- b. Unidirectional flow of the surrounding fluid. Flow rate is controlled by a single piston displacement pump.



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We have employed interference reflection microscopy (IRM) to visualize the sites of close contact between gliding bacteria and glass substrata on which they adhere and glide.

IRM: A freshwater isolate, *Cytophaga* U67, has been a model system in our IRM studies; it glides actively and for extended periods on glass, unlike *F. maritimus*. These observations indicate that a bacterium can glide with only a small portion of the cell surface in close association with the substratum. The sites of association are dynamic; they change in position and number as the bacterium translocates. Individual sites of close association move relative to substratum, casting doubt on one recent motility model involving adhesive sites associated with a track system in the bacterial cell wall. IRM also reveals that rotation around the long axis of the bacterium is coupled to translocation, except when a curved cell moves in a curvilinear direction. This study has been submitted for publication.

Monoclonal Antibodies Raised Against Slime: Monoclonal antibodies directed against slime should provide powerful tools for analysis of the role(s) of this extracellular material in adhesion and motility. It may also be possible to identify function domains of the slime complex with monoclonals. We have used hybridoma technology to prepare monoclonal antibodies directed against whole, slimy *F. maritimus* cells. It would have been preferable to use isolated slime as the antigen. However, until we are successful in isolating this material (see above), this expedient was necessary. We predicted that the outer, slimy surface of whole bacteria would elicit a response from the mouse immune system. The 11 ELISA-positive clones that we have generated are currently being tested further.

Related, ongoing research:

Cell Envelope Polypeptides that may Mediate Adhesion: Studies in collaboration with R. Bloodgood (U. of Virginia) on *Cytophaga* U67 have identified a variety of cell envelope polypeptides that make physical contact with glass substrata. These polypeptides were labelled on actively gliding cells, using an immobilized radio-iodination catalyst (IODO-GEN). This bacterium has been reported to produce little slime. Thus, direct contact of the outer membrane with the substratum is likely. By comparing the pattern of labelled polypeptides from adhesion mutants with those from the wild-type, we have identified at least two that may be involved in adhesion. The mutants were isolated by serial enrichment for bacteria with relatively hydrophilic surface properties, using a hexadecane/aqueous medium partitioning protocol. One of these polypeptides is "shifted" in molecular weight from 116 KD in the wild-type to 114 KD in the mutants. The second, an 86 KD polypeptide, is iodinated in the adhesion mutants but only labelled weakly, if at all, in the wild-type. We speculate that a change in the surface topology of the mutants masks adhesion sites with this 86KD polypeptide. A revertant of one of the adhesion mutants demonstrates a wild-type labelling pattern.

We have also used biotinylation of the cell surface to compare U67 with its adhesion mutants. A manuscript describing this study is in preparation.

We are currently comparing the fine structure of the surfaces of *Cytophaga* U67 and its adhesion mutants using freeze-fracture techniques. This study is in collaboration with D. Pumplin of the University of Maryland's School of Medicine.

Effect of Temperature Shifts on Adhesion and Motility. Several years ago we observed that 25° - grown *Cytophaga U67* cells, when shifted to a 4° environment, stopped gliding. Only after several hours of growth at 4°C did motility re-commence. In contrast shifting 4° grown cells to 25° had no inhibitory effect on motility. Our recent studies demonstrate that the temperature down-shift does not affect adhesion.

We have examined changes in fatty acid composition of the cytophagae during these temperature shifts (collaboration with C.W. Moss of C.D.C., Atlanta). Our data indicate that bacteria downshifted to 4°C undergo a homeoviscous adaptation, involving replacement of some saturated with unsaturated fatty acids, before motility competence is achieved. A manuscript describing this study is in preparation.

Work Plan (Year 2): We will continue our characterization of slime production and its role in adhesion and motility of *Flexibacter Maritimus* and selected other aquatic gliding bacteria. Additional mutants in slime production and adhesion will be isolated by transposon mutagenesis. We will attempt to introduce transposable elements into recipient bacteria by electroporation and tri-parental matings. Adhesion properties of mutants and wild-type bacteria will be characterized using the adhesion assays described above.

The slime of *F. maritimus* and selected mutants in adhesion and slime production will be characterized rheologically (see part 2) and biochemically. The polysaccharide structure analysis will be undertaken by I.W. Sutherland (University of Edinburgh). Slime polypeptide composition and function will also be examined. We will utilize anti-slime monoclonal antibodies in the structural and functional analysis of this extracellular material.

Our long-term goal is to use experimental and theoretical approaches to elucidate the function(s) of extracellular slime in adhesion and gliding motility.

Progress Report on the Mechanism of Gliding and Adhesion

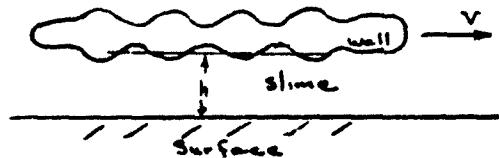
Investigators: W.H. Schwarz - Johns Hopkins University
Abdul Siddiqui - Penn State Univ.-York

Research Objective: To develop mathematical models for the mechanism of propulsion of gliders and to study the relationship of the rheological properties of slime to adhesion and translocation.

Introduction and Background

The mechanism used by gliders to translocate on surfaces has not been unequivocably elucidated. In fact, different methods of propulsion probably have been evolved by organisms. However any proposed mechanism of motility must be consistent with some, or all, of the following observations: 1) rapid reversals of direction; 2) adhesion to the surface by slime that forms a sheath around the cell; 3) helical rotation of the organism; 4) micro-particles in surrounding media adhere to and are transported on the surface of the glider; 5) the lack of motility without the presence of a surface and, 6) the cylindrical shape of gliders.

A mechanism for motility has been proposed by Halfen and Cartenholz from their observations of Oscillatoria princeps (and other blue-green algae) and also by Humphrey, et al. from their experiments on Flexibacter BH3. Using electron microscopy they concluded that the walls of the glider undulate in the form of an axial traveling wave (see sketch below).



Actually the wave observed on the Oscillatoria travels obliquely to the axis (helically) and thereby imparts a torque and subsequent rotation. This aids in the smoothing and the uniform removal of the sheath, and tends to keep the organism tracking in a straight line (on average) even though the organism may be curved.

Progress

During the past year, we have developed the mathematical equations that describe the motion of the exuded slime layer that is driven by the traveling wave. This hydrodynamic theory uses the principles of the conservation of mass and linear momentum, and appropriate initial and boundary conditions to obtain a deterministic set of differential equations. Their solution obtains the velocity field of the slime, from which the local stresses at the boundaries can be calculated. Integration of the stresses determines the force (F) that propels the microorganisms in terms of (for our model): the thickness of the slime layer, the rheology of the slime, the amplitude wave length and frequency of the propagating wave and the geometry length scales of the organism. The translocating velocity (V) is determined by a balance between the propulsive force and the resistive forces caused by the drag on the self-propelled glider, and the viscous dissipation by the induced motion in the slime boundary layer.

If calculated values of the translocating velocity compare with the observed values, then the model is plausible. Also, since the power (P) expended by the organism is determined by the relation: $P = V \cdot F$, computed values can be compared to estimates of the energy that is produced by the organism.

Methods of Solution

The resulting set of partial differential equations are non-linear and difficult to solve. However, for the conditions that dimensions (d) and velocities (V) are small, and the fluid is very viscous, the dimensionless parameter: $\sqrt{d} \rho / \mu$ (ρ is the density) is small, and the equations can be simplified, i.e., the non-linear inertia terms are neglected (called the creeping motion condition). We are currently developing solutions to this reduced problem by the method of regular perturbation expansions.

In order to solve these equations, it is necessary to prescribe the motion of the wall of the organism, and the surface (considered to be rigid). Also we must specify the adherence of the slime to those boundaries. We have assumed that the slime sticks to both surfaces, therefore the displacement of the slime at the boundary is the same as the boundary (the classical no-slip condition). We note however, that experiments indicate that the motility of certain gliders are affected by the type of surface (e.g. glass, agar, etc.). Our hydrodynamic theory can account for this effect by introducing slip at the wall.

Rheological Properties of Slime

In order to model the glider by hydrodynamical equations, we must relate applied stresses to the deformation (motion) of the slime by a rheological equation. A number of relations have been previously proposed and have been found to be useful for modeling other hydrodynamics problems. We have adopted the constitutive functional equation for a "simple fluid" that describes viscoelastic materials with memory. For small enough rates of deformation relative to the non-Newtonian nature of the material, the functional can be represented by ordinary functions of kinematic variables (Rivlin-Ericksen tensors) to some order. We have used the third-order approximation.

Preliminary measurements on slime obtained from the culture media of various gliders indicate the material to be a shear-thinning viscoelastic fluid. However, the mixture contains growth media and cells and may not be representative of the slime in situ.

The importance of rheological data cannot be overstated, because of their need for our mathematical models and also the material is a component of compliant biofilms that form on surfaces (ships) in a marine environment. Rheological knowledge will permit fabrication of a synthetic material with the same compliant properties that can then be studied with respect to its interaction with the turbulent boundary layer.

Plan of Attack - Year 2

1. Do the calculations for the traveling wave model that we have developed during the first year.
2. Theoretically explore the effect of using different expressions for the motion of the wall of the glider (elastic versus inelastic).
3. Measure the rheological properties of the slime obtained from the cultures obtained by Dr. Burchard.
4. Examine the effect of using other constitutive equations for the rheological properties of the slime.
5. Determine the range of validity of the lubrication theory approximation by determining solutions to the full problem.
6. Examine numerical methods of solution of the hydrodynamical equation.
7. Consider other models of propulsion.